The Mucoprotein of the Fat/Plasma Interface of Cow's Milk. II. Immunochemical Characterization[1]

The sialic acid-containing mucoprotein from the fat/plasma interface of cow's milk functioned as a strong antigen. The mucoprotein preparation induced formation of antibodies in rabbits to the mucoprotein and to at least two contaminating proteins from whey. Absorption of the antiserum with a preparation of whey proteins resulted in a homogeneous antibody system specific for the mucoprotein. This mucoprotein was distinguished immunologically from other, previously recognized proteins of milk.

INTRODUCTION

In the preceding paper Jackson, Coulson, and Clark (1) described some of the chemical and physical characteristics of a mucoprotein derived from the surface film of fat droplets from milk. Free-boundary electrophoretic data indicated that the mucoprotein was essentially homogeneous with respect to surface-charge density. In the analytical ultracentrifuge, this mucoid displayed a single boundary in a low gravitational field; convective disturbances were encountered in high gravitational fields. The composition of the mucoprotein was different from the major milk proteins with respect to the presence of sialic acid, hexose, and hexosamine.

Immunochemical methods are proving increasingly useful for exploring the homogeneity or heterogeneity of native proteins. In general these methods are more sensitive than physical methods for detecting heterogeneity. This report describes the application of immunochemical methods to the characterization of the mucoprotein and its differentiation from other, recognized proteins of cow's milk.

[1] Presented at the 140th Meeting of the American Chemical Society, Chicago, Ill., September 1961.

[2] Eastern Utilization Research and Development division, Agricultural Research Service, U. S. Department of Agriculture.

EXPERIMENTAL

Milk Proteins

α-Lactalbumin was isolated and crystallized four times by the method of Gordon and Ziegler (2). β-Lactoglobulin was crystallized four times by the method of Larson and Jenness (3). α-Casein was prepared by the Warner (4) method as modified by McMeekin *et al.* (5). β-Casein was prepared from the filtrate of the α-casein separation by the method of Warner (4). κ-Casein was prepared as described by Fox (6). Pseudoglobulin and euglobulin were prepared by the methods of Smith (7). Preparation of the mucoprotein was described in the preceding paper (1).

Whey proteins were separated from the caseins by subjecting fresh skim milk to a gravitational field of 44,330 × *g* for 120 min. The calcium–caseinate pellets were discarded, and the greenish yellow supernatant containing the whey proteins was lyophilized. This preparation undoubtedly contained some of the smaller casein micelles and a trace of fat, containing interfacial components.

Production of Rabbit Antiserum

Three rabbits weighing 2.5–3 kg. were immunized to the mucoprotein by a series of 12 weekly, subcutaneous injections of antigen (5 mg. protein/dose) in Freund's adjuvant (8). Antibody production was maintained by a series of four daily, intravenous injections of 1 ml. of a 0.5% solution in

saline once every 2 weeks. The rabbits were bled from the heart after the seventh week on this schedule, and then every 2 weeks for six bleedings. The sera from all bleedings were pooled.

Immunoelectrophoretic Analyses

The techniques as described by Grabar (9) and the micro technique of Scheidegger (10) were used. There was no significant difference between the patterns of the mucoprotein obtained with the two methods. Special Agar-Noble[3] was used without further purification.

Ouchterlony Analyses

Ouchterlony plates (11) were prepared by pouring hot, 1.5% agar into 100 × 15 mm. Petri dishes and immediately pouring it out. After this thin layer had cooled, a second layer of agar was poured to a depth of 3 mm. Wells for antigen and antiserum were formed in this second layer by pouring the agar around metal molds.

In Vitro Anaphylactic Studies

Virgin female guinea pigs weighing 200–250 g. were sensitized by a single intraperitoneal injection of the protein dissolved in saline. The sensitizing doses of the different preparations were: α-lactalbumin 3.2 mg., β-lactoglobulin 1.0 mg., α-casein 20 mg., β-casein 3.2 mg., and mucoprotein 0.4 mg. For the first four proteins listed, the sensitizing dose was equivalent to 2–3 times the median sensitizing dose (the dose that fatally sensitizes 50% of the animals). The sensitizing dose for the mucoprotein was about 20 times its median sensitizing dose. The incubation period ranged from 3 to 4 weeks.

The Schultz-Dale (12, 13) experiments were conducted by the technique previously described (14). Each of the two uterine horns from a sensitized guinea pig was cut into two strips. One strip from each horn was stored in oxygenated Tyrode's solution. The other strips were mounted in two adjacent Dale baths. When the muscles were completely relaxed, the challenge dose was introduced and the responses were recorded on the kymograph. The muscles were challenged first with a dose of heterologous protein containing 10 μg. of protein nitrogen (N). If a positive response was obtained, testing was discontinued with that strip. When no response occurred, the strip was subsequently challenged with another heterologous protein or with a 1-μg. protein N dose of homologous protein. The two strips previously stored in Tyrode's solution were then used to test the remaining proteins. Control tests of proteins with uterine muscles from nonsensitized guinea pigs were negative at the 10-μg. N level, but some showed nonspecific contractions at the 100-μg. N level.

[3] Purchased from Difco Laboratories. Mention of products in this paper does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

Precipitin Studies

The quantative precipitin determinations were conducted by the method of Heidelberger and Kendall (15) as described by Kabat and Mayer (16). The analyses were carried out by adding 1.0 ml. of 1:2 diluted antiserum to each of a series of tubes containing increasing amounts of mucoprotein in 1 ml. of buffered saline. The contents were immediately mixed and stored 1 hr. at 37°C. and then 5 days at 5°C. The precipitates were centrifuged at 2°C. and washed twice with cold saline. Nitrogen contents of the precipitates were determined by the micro-Kjeldahl method. The results are recorded on the basis of antibody N/ml. antiserum.

RESULTS

The lower half of Fig. 1 shows a typical immunoelectrophoretic pattern of the mucoprotein preparation with its antiserum. Three bands of precipitation developed. The main band, representing the reaction of the mucoprotein with its antibody, formed within 24 hr. Later, after 48–96 hr., two faint precipitin bands appeared, one on each side of the main band. These faint bands revealed at least two different protein contaminants associated with the mucoprotein.

With the Ouchterlony technique, the mucoprotein preparation showed only one faint band in addition to the main precipitin band of the mucoprotein. The faint band appeared between the main band and the antiserum well.

The Ouchterlony technique was used to compare the mucoprotein with other proteins of milk. Among the whey proteins analyzed by this method were β-lactoglobulin, α-lactalbumin, bovine serum albumin, pseudoglobulin, and euglobulin. Also compared were: α-casein, β-casein, κ-casein, and a γ-casein-rich fraction. None of these proteins formed precipitin lines with the mucoprotein antiserum. The κ-casein fraction contained sialic acid but formed no precipitin band with the mucoprotein antiserum.

A precipitin band appeared when the

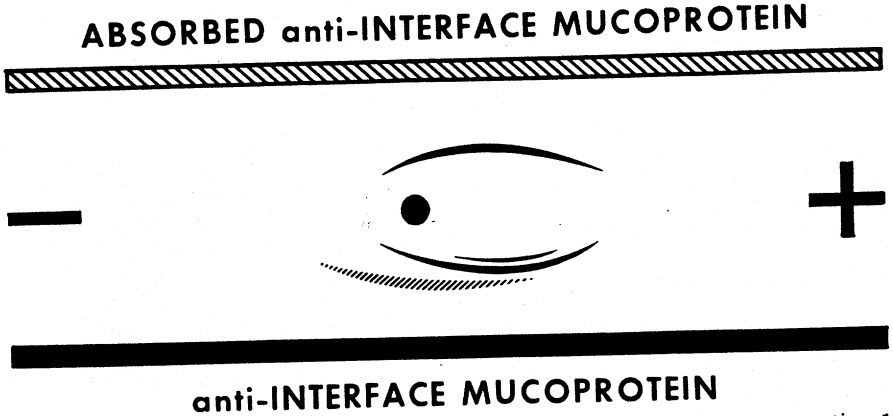

FIG. 1. Immunoelectrophoretic separation of the mucoprotein. Protein concentration 1%, Veronal buffer pH 8.2, ionic strength 0.05, time 5400 sec., 90 v., and current 60 ma.

preparation of whey proteins was compared with the mucoprotein against mucoprotein antiserum. This band merged with the faint band of the mucoprotein preparation in a reaction of identity. The common component, which was not identified, may be a whey constituent or an interfacial component. Skim milk from which the whey proteins were prepared invariably carries traces of smaller fat globules containing interfacial components.

Table I records the *in vitro* anaphylactic responses of four major milk proteins and the mucoprotein. The sensitized tissues were challenged with 1 μg. protein N of the homologous protein or with 10 μg. protein N doses of the heterologous proteins, except as noted in Table I.

TABLE I

IN VITRO ANAPHYLACTIC REACTIONS TO MILK PROTEINS

Animals sensitized to:	Anaphylactic response[a] to:				
	α-Lactalbumin	β-Lactoglobulin	α-Casein	β-Casein	Mucoprotein
	Reaction ratios[b]				
α-Lactalbumin	5/5[c]	5/5[c]	1/5	0/5	0/5
β-Lactoglobulin	1/7	7/7[c]	2/7	0/7	0/7
α-Casein	0/10	5/10	6/10[c]	6/10	0/10
β-Casein	0/6	2/6	5/6	6/6[c]	0/6
Mucoprotein	0/9	1/9	0/9	0/9	9/9[c]

[a] Challenge doses were 10 μg. protein N in a 50-ml. bath, except as indicated.

[b] Reaction ratio = $\frac{\text{No. of animals reacting}}{\text{No. of animals tested}}$.

[c] Challenge dose was 1 μg. protein N.

Five animals were sensitized to α-lactalbumin. Tissues from all five responded to challenge with 1 μg. protein N of α-lactalbumin and, likewise, all five responded to the same dose of β-lactoglobulin. One of the five animals responded to α-casein. No responses were recorded to β-casein or to the mucoprotein.

Tissues from all seven animals sensitized with β-lactoglobulin reacted to the homologous protein, one reacted slightly to α-lactalbumin, and two gave slight reactions to α-casein. None reacted to β-casein or to the mucoprotein.

Ten animals were sensitized with 20 mg. α-casein. Nine of these animals were sensitive to one or more of the five proteins, but only six of the ten reacted to the homologous preparation. Six responded to test with β-casein and five reacted with β-lactoglobulin. None responded to test with either α-lactalbumin or the mucoprotein.

Six animals were sensitized with β-casein, and all reacted to challenge with β-casein. Five of the animals reacted also with α-casein, and two reacted with β-lactoglobulin. No reactions were observed with α-lactalbumin or with the mucoprotein.

All nine animals sensitized with the mucoprotein responded to test with the homologous protein. Only one heterologous reaction was observed in this group. This response to a 10-μg. protein N dose of β-lactoglobulin was confirmed by a similar

response to challenge with 1 μg. protein N of β-lactoglobulin on another uterine strip from the same animal and was thus shown to be a specific response.

Table II shows the quantitative precipitin reaction of the mucoprotein with its antiserum. The absence of an equivalence zone, observed in the tests on the supernatants from the precipitates, showed that the mucoprotein was serologically heterogeneous and confirmed the results of the immunoelectrophoretic analysis.

It seemed pertinent to determine whether antibodies to the contaminating proteins in the mucoprotein antiserum could be removed by absorption with the mixture of whey proteins. Table III records the precipitin reactions of the whey protein preparation with the mucoprotein antiserum. With increasing additions of whey proteins, the total nitrogen precipitated rose sharply to a maximum of 0.195 mg. when 0.2–0.3 mg. of whey protein N was added to 1 ml. of the serum. Accordingly, for preparation of absorbed serum, 0.26 mg. of whey protein N/ml. was added to a suitable volume of antiserum. After incubation and storage in the refrigerator in the usual manner, the precipitate was removed by centrifugation.

The immunoelectrophoretic pattern of the mucoprotein with the absorbed antiserum is shown in the upper half of Fig. 1. The results show that antibodies for the contaminating proteins were removed by reaction with some components of the whey preparation.

Table IV presents the results of the precipitin analysis of the mucoprotein with the absorbed serum. The reaction behaved like a system in which at least one of the reactants was homogeneous. There was a broad equivalence zone between 0.14 and 0.20 mg. of mucoprotein N added. Within this zone the antibody N precipitated was essentially constant. Comparison of the results in Table IV with those in Table II shows that about 10% of the antibody N was removed by

TABLE II

PRECIPITIN REACTION OF MUCOPROTEIN WITH ANTISERUM

Mucoprotein N added	Total N pptd.	Antibody N pptd.[a]	Ratio AbN: AnN in ppt.	Tests on supernatants	
				For antibody	For antigen
mg./ml.	*mg./ml.*	*mg./ml.*			
0.02	0.190	0.170	8.5	++++	±
0.04	0.290	0.250	6.3	++++	±
0.06	0.373	0.313	5.2	+++	+
0.08	0.454	0.374	4.7	+++	+
0.10	0.510	0.410	4.1	+	+
0.12	0.560	0.440	3.7	±	+
0.14	0.598	0.458	3.3	−	+
0.16	0.629	0.469	2.9	−	+
0.18	0.654	0.474	2.6	−	+
0.20	0.671	0.471	2.4	−	+
0.24	0.709	0.469	2.0	−	+
0.28	0.724			−	++
0.32	0.712			−	++++

[a] These are approximate values because the antigen was not completely precipitated.

TABLE III

PRECIPITIN REACTION OF WHEY PROTEINS WITH MUCOPROTEIN ANTISERUM

Whey protein N added	Total N pptd.	Tests on supernatants	
		For antibody	For antigen
mg./ml.	*mg./ml.*		
0.05	0.091	+++	±
0.10	0.129	+++	+
0.20	0.195	+	+
0.26	0.196	+	+++
0.30	0.194	+	+++
0.40	0.186	+	+++

TABLE IV

PRECIPITIN REACTION OF MUCOPROTEIN WITH ABSORBED ANTISERUM

Mucoprotein N added	Total N pptd.	Antibody N pptd.	Ratio AbN: AnN in ppt.	Tests on supernatants	
				For antibody	For antigen
mg./ml.	*mg./ml.*	*mg./ml.*			
0.02	0.143	0.123	6.2	++++	−
0.04	0.245	0.205	5.1	+++	−
0.06	0.333	0.273	4.6	+++	−
0.08	0.401	0.321	4.0	++	−
0.10	0.478	0.378	3.8	+	−
0.12	0.531	0.411	3.4	±	−
0.14	0.563	0.423	3.0	−	−
0.16	0.599	0.439	2.7	−	−
0.18	0.603	0.423	2.4	−	−
0.20	0.615	0.415	2.1	−	−
0.24	0.651	0.411	1.7	−	±
0.28	0.660			−	+
0.32	0.671			−	++

absorption with the whey proteins. The ratio of antibody N to antigen N in the equivalence zone ranged from 3.0 to 2.1.

The data in Tables II and IV were tested for conformity to the two equations introduced by Heidelberger and Kendall [cf. (16)] to describe the course of other antigen–antibody reactions by plotting the ratio of antibody N to antigen N in the precipitates against the amount of antigen N added and against the square root of antigen N added. The latter plot gave a straight line for the data obtained with the absorbed serum through the zone of antibody excess and continuing through the equivalence zone. The data obtained with the unabsorbed serum did not conform to a straight line with either method of plotting.

DISCUSSION

Most protein preparations, even those prepared by crystallization, are associated with serologically detectable impurities derived from the mixture from which they are prepared. These impurities persist through a number of recrystallizations [e.g., ovalbumin (17) and β-lactoglobulin (18)]. The results of the Schultz-Dale tests recorded in Table I of this report show that purified milk proteins, α-casein, β-casein, α-lactalbumin and β-lactoglobulin, are contaminated with each other or with other proteins in common. These results do not agree with the results of Ratner *et al.* (19) who reported that α-casein, β-lactoglobulin, and α-lactalbumin were immunologically homogeneous as determined by gross anaphylaxis and by the Schultz-Dale test.

A mucoprotein isolated from the surface of milk-fat globules was associated with at least two immunologically distinct species of molecules. These were present in quantities sufficient to stimulate antibody formation in rabbits immunized with the aid of Freund's adjuvant. Antibodies to the associated proteins were absorbed from the mucoprotein antiserum by a whey protein preparation to yield a homogeneous antibody system. This procedure removed about 10% of the antibody N, part of which was probably precipitated by traces of mucoprotein contained in the whey preparation.

As judged by the Schultz-Dale tests, the mucoprotein appears to be more nearly immunologically homogeneous than the usual milk protein preparations. The concentration of the major antigen in the mucoprotein preparation is being studied by Dr. Jean Harris, in Dr. E. L. Becker's laboratory, by a quantitative gel-precipitin technique (20, 21).

The data in this report show that the mucoprotein is distinct from other, recognized proteins of cow's milk.

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